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THE BIOLOGICAL EFFECTS OF CROCIDOLITE ASBESTOS AND SILICON CARBIDE FIBERS ON MAMMALIAN CELLS IN CULTURE

A THESIS

PRESENTED FOR THE

MASTER OF SCIENCE

DEGREE

THE UNIVERSITY OF TENNESSEE, KNOXVILLE

JACQUELINE ALICE JORDAN

AUGUST 1991

DEDICATION

This thesis is dedicated to my loving and caring family, especially my mother, Maggie. I will always remember the love and support (financial and personal) you have given me throughout my academic career. Thanks for always being there when I needed you.

Love,

Jackie

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ABSTRACT

To study the mechanisms associated with the carcinogenicity of natural and man-made fibers/whiskers at the cellular level, mouse fibroblast cultures were exposed to 5 μg/cm2 crocidolite asbestos (0.36 μM in diameter) and two different sizes of silicon carbide whiskers (0.8 and 1.5 μM in diameter). After 48 hours of exposure, 60 to 90 percent of metaphase spreads had chromosomes that were entangled with, attached to, or lying adjacent to fibers. Over 40 percent of isolated nuclei contained fibers, and were seen inside of, or attached to various parts of the nucleus (i.e. nuclear membrane, nuclear matrix, DNA, chromatin). Total DNA and chromosomal content were also determined up to 20 generations post exposure to test materials. After 10-12 generations postexposure (6-8 passages), all fibers/whiskers induced a 40 to 75 percent increase in cellular DNA. There was also a significant increase in chromosome number per cell 18 generations post exposure to fibers/whiskers, as compared to control. This increase in DNA and chromosome content could possibly provide information on the cellular mechanism of toxicity associated with the exposure to fibrous materials. These quantitative in vitro tests may also be useful as a tool to suggest the long-term genetic effects of a potential carcinogen.

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1. INTRODUCTION

OVERVIEW

Occupational, as well as non-occupational, exposure to asbestos fibers may cause pulmonary fibrous (asbestosis), pneumoconiosis, and even cancer (bronchial carcinoma and mesotheliomas) (Stanton, *et al* 1977; Lee, *et al* 1979; Norseth, 1980; Saracci, 1985; Brody, 1986; Lippman, 1988; Mossman, *et al* 1989; Jaurand, *et al* 1989). The cellular mechanisms responsible for these toxic effects have not been determined. Epidemiological and animal studies conclude that the chemical and physical properties of the fibers are critical determinates (Stanton, *et al* 1977; Lippman, 1988; Lipkin , 1980).

Awareness of these toxic effects, primarily to the industrial users of asbestos, has resulted in the decline in the production of these materials. This decline has resulted in the search for other natural fibers (zeolite) and the increased production of man-made fibers (single cystal whiskers or fibers, carbon fiber, spun ceramics) to successfully replace asbestos for many of its industrial purposes (Saracci, 1985; Jaurand, *et al* 1986). For technological reasons, the majority of these substitutes must maintain structural characteristics similar to asbestos, and it is now believed that these similarites could cause them to be equally as toxic.

RESPIRATORY DEFENSE AGAINST INHALED PARTICLES

Under normal conditions, the respiratory system possess several lines of defense against the inhalation of foreign particles. Large particles, over 5 to 10

μM in diameters, are trapped in the hairs and mucus of the nasophargnx, and thus are unable to enter and damage the airways. Respiratory clearance at this level, however, is significantly affected by mouth breathing rather than nose breathing. Mouth breathing may reduce clearance at this level by 50% (Asgharian *et al*, 1988). Particles that range from 1 to 5 μM in diameter are deposited into the tracheobronchial region. Clearance from this region, as well as the nasophargnx region, is primarily by mucociliary transport. Cilia from epithelial cells lining the respiratory tract, sweep particles trapped in mucous into the oral cavities, where they are swallowed or excreted. Very small particles, usually less than 1 μm in diameter, travel deep into the alveolar regions of the lungs where they encounter a large population of mobile phagocytic cells, referred to as macrophages. These macrophages are responsible for engulfing these small particles and transporting them to the upper levels of the respiratory tract for clearance. However, the mechanism for how this is accomplished is unknown (Gross, *et al* 1986; Klassen *et al*,1984).

Experimental studies, human and animal, have proven that some small particles, primarily fibrous particles like asbestos, are not removed by the clearance mechanisms of the respiratory system (Asgharian, *et al* 1988; Timbell, 1984; Gross, *et al* 1984, Chamberlain, *et al* 1985; Lippman, 1988). Lemaire, *et al* (1989) concluded the localization of these materials is dependent on fiber diameter and secondary its length. Fibers smaller than 5 μM and of up to 2 μMs in diameter will achieve greater alveolar penetration than wider fibers that may be intercepted by smaller bronchioles. Timbell, *et al* (1983) suggest both short and long fibers align with the direction of airflow and therefore are able to reach the peripheral regions of the lung. The shorter fibers are cleared

by macrophages, but the longer, thinner fibers remain in the lung interstitum (Brody, 1986). These fibrous materials accumulate in a dose-dependent manner in the bronchial and alveolar regions of the respiratory tract, where they exert severe toxic effects (Brody, 1986; Lemaire, 1986; Timbell, 1983).

BACKGROUND

Asbestos, refers to a large group of hydrated silicates that possess unusually high tensile strength, flexibility, and chemical and physical durability. It occurs naturally and has a distinct fibrous crystalline structure, which is responsible for its unique properties. Chrysotile is the most important commercially and represents some 95% of the total usage. Other minerals marketed as asbestos includes amosite, crocidolite, anthophyllite, tremolite, and actinolite. All types of asbestos materials have been used in several forms since prehistoric times. These uses include the reinforcement of clay pottery, wicks in the gold lamps of goddess, and in cloth to retain the ashes of the dead after cremation. One of the earliest dramatized use of asbestos was in 1725 when Benjamin Franklin talked of his purse made of asbestos fibers.

It wasn't until 1890, with the industrial revolution, that there was a significant increase in the production of asbestos. Several uses of asbestos were found and therefore exploited commercially. Some of the industrial uses of asbestos include fireproofing, gas masks (World War I and II), floor tiles, friction materials (brake linings and clutch pads), and in the reinforcement of cement (cement pipes) (Gross, *et al* 1984, Mossman, 1990). The demand for asbestos continued to increase until the early 1970's. It was during this time that

the severe toxic effects of asbestos were recognized by the public. Since then, there has been a significant reduction in the use and production of asbestos materials. To date, other countries (Turkey, Nertherlands, South Africa) still continue to produce significant quantities of these materials.

Since the recognition of the toxicity associated with the industrial exposure to asbestos fiber, experimental studies have been conducted to determine the specific biological parameters involved. King, *et al* (1946) was one of the first researchers to suggest the importance of fiber length in the production of pulmonary fibrosis (asbestosis). Animals injected with long fibrous materials, like chrysotile asbestos, developed peribronchial fibrosis. Since then several forms of fibrous materials, including asbestos, silicon carbide, and glass have been examined. Stanton, *et al* (1977, 1981) correlated particle dimension and geometry with the carcinogenicity for 72 fibrous and non-fibrous minerals of wide range of chemical and structural properties. The fibers with the highest tumor probability were those ≤ 1.5 μM in diameter and $\geq$ 8 μM in length. The fibers in this range lie free in the interstial spaces of the lungs, while shorter fibers are removed by alveolar macrophages. Also, milling these fibers, or converting to a non-fibrous form, significantly reduced their biological effects. Small glass fibers, for example, are potent in transforming cells <u>in vitro</u> as are various forms of asbestos fibers. The powdered form of the same materials have very little or no cytotoxic, genotoxic or carcinogenic ability (Sincock, 1977). From this observation, Stanton, as well as others, concluded the physical properties, rather than chemical nature of asbestiforms and man-made fibers/whiskers were responsible for the development of cancer (Pott, Chamberlain, 1978).

Asbestos exists in a variety of forms which differ in the severity of their toxic effects. Huang, *et al* (1982) concluded that chrysotile is approximately 10 times more cytotoxic than either amosite or crocidolite and more than 100 times more toxic than glass. The type of fiber, and its persistence in lung tissue, is also critical in determining the type of cancer produced. Chrysotile, accounts for over 95% of the industrial use of asbestiform materials. The other 5%, amphibole fibers, are thought to be the primary cause of mesotheliomas in exposed workers. Even though, the workers are predominantly exposed to chrysotile, amphibole fibers are more persistent in lung tissue. (Lippman, 1988; Mossman, 1989).

In the last few years a variety of fiberform materials, such as silicon carbide whisker or fibers, have entered the marketplace. Silicon carbide whiskers are a new commerical product currently being manufactured in the United States and Japan. It is currently used as a heat exchanger, in large radiant tubes, as a semiconductor, and largely in the reinforcement of ceramic and metals (Sheppard, 1987). It is produced by mixing an organic carbon source with highly purified crystalline silica, and then heating this mixture to 2000-2200 C. During this process large quanitites of carbon monoxide and smaller quanitites of sulfur dioxide and airborne particulates that contain SIC fibers and crystalline silica are released into the air (Smith, *et al* 1984). For the first 7 to 8 years of market development, the issue of health and safety of this material was largely ignored, in spite of obvious similarities to asbestos (American Matrix, 1986). Stanton, *et al* (1981), concludes that the length to diameter ratio of SIC fibers causes it to maintain a 100% probability to produce neoplastic changes (mesothioliomas). Recent intratracheal instillation studies from this lab, using

experimental animals, suggest long-term exposure to SIC fibers produce pulmonary fibrosis and granulomas (pre-cancerous changes). Because of the increased use of these materials as a substitute for asbestos, these severe toxic effects deserve serious attention.

PURPOSE

The molecular and cellular mechanisms associated with both the fibrogenic and carcinogenic action of asbestos and man-made fibers have not been determined. Epidemiological studies as well as whole animal studies are not able to provide sufficient information on the toxicity of these materials at the cellular level. A variety of in vitro techniques are now being employed to study the carcinogenicity of solid state materials (Mossman, 1987; Jaurand, *et al* 1989; Wang, *et al* 1987; Sincock, 1983; Huang, *et al* 1978; Chamberland, *et al* 1978). In vitro tests might well detect genotoxic carcinogens but positive responses in vivo will go largely undetected. In vitro tests followed by a short term in vivo tests may confirm genotoxic endpoints. The purpose of this research was to compare the cellular mechanism of toxicity of silicon carbide fibers to that of crocidolite asbestos, using mammalian cells is culture. This study includes the quantitative analysis of DNA and chromosome content postexposure to fibers. The results conclude asbestos and man-made fibers exert similar cellular effects.

2. MATERIALS AND METHODS

TEST MATERIALS

Two silicon carbide whisker/fiber (SiCW) samples were used in this study, one of 0.8 μM diameter (SiCW-1) manufactured by Tateho, Japan, and one of 1.5 μM diameter (SiCW-2) manufactured by American Matrix, Inc., Knoxville, TN. A sample of crocidolite asbestos was kindly provided by Dr. L.W. Ortiz of Los Alamos National Laboratory, Los Alamos, NM. Dimensional characteristics of these materials were determined from measurements made on scanning electron micrographs, see Table 1. Samples of each test material were suspended by sonication in phosphate buffered saline (PBS) at a concentration of 1.0 mg/ml and sterilized by autoclaving before use. These materials are not soluble and settle to the surface of test plates, and since depths of fluid in various culture vessels varies, concentration is always expressed as mass of material per surface area.

CELL CULTURE AND TREATMENT PROCEDURE

Balb/3T3 clone A31 embryonic mouse fibroblast cells were obtained from American Type Culture Collection, Rockville, MD. This cell line was developed by S.A. Aaronson and G.T. Todaro in 1968 from disaggregated 14-17 day old BALB/c mouse embryos. It is hypotetraploid with a stemline of 2n equal 40. It has been used in studies relating to in vitro properties associated with tumorigenicity and contact inhibition. In this study, the mouse cell

TABLE 1. DIMENSIONAL CHARACTERISTICS OF TEST MATERIALS

Material	Diameter*	Length	Aspect Ratio (LxD^{-1})
SiCW-1	0.8 (0.3)	18.1 (14.3)	23.3 (18.7)
SiCW-2	1.5 (0.6)	19.0 (11.0)	15.3 (11.2)
Asbestos	0.36 (0.23)	4.3 (0.2)	14.3 (3.8)

* dimesions given in microns

( ) standard deviation

cultures were maintained as described previously (Vaughan, *et al) 1991.* Briefly, cultures are grown in 25cm2 flask and maintained in Minimal Essential Medium with Earles salts (MEM) supplemented with 10% calf serum and antibiotics (penicillin-streptomycin) at 37 C in an atmosphere of 5% co2 in air. Cultures are divided 1:4 by trypsinization (0.25%) in PBS upon reaching 80% confluence, and allowed to attach for 24 hours; this equals 1 passage of 2 generations. Upon reaching 40% confluence, the cell cultures are exposed to 5 ug/cm2 concentration of crocidolite asbestos or silicon carbide fibers for 48 hours, remembering not to exceed 80% confluence. This is determined by viewing the cultures under an inverted phase microscope. The overall area between the each cell determines the confluency of the cultures. Cultures with cells in total contact with one another are considered 100% confluent. Mouse fibroblasts may spontaneously give rise to continous cultures. If cells are routinely kept from reaching complete confluence this diminishes the probability of mouse fibroblast cells from giving rise to a continous cell line (Freschney, 1989). Control culture are treated with PBS and maintained concurrently. Trypsin and components of the culture medium were obtained from GIBCO, Grand Island, NY. Cell numbers were determined with a Coulter Electronic Cell Counter (model ZM) or by hemocytometer.

DNA DETERMINATION

In this analysis, the cells are grown in 75cm2 flask, and at every other passage (4 generations), harvested, counted, and frozen for later determination of total cellular DNA content, using the colorimetric method of Keck (1956).

The cells are hydrolyzed at 90 C in 10% TCA to break down cell membranes , which results in the release of the cellular components. The free nuclei acids are reacted with 0.06% Indole, and the bonding between the two produces a pinkish color. The samples are then extracted with amyl acetate to remove RNA , leaving the DNA in solution. DNA concentration is determined colorimetrically and compared to that of a calf thymus DNA control. It is expressed as ug/cell or as a percent of control. Total DNA content is determined every 4-6 generations postexposure to fibers. Cultures are followed up to 20 generations; the untreated cultures maintained concurrently. The average DNA content from generations 10-20 are reported here.

METAPHASE PREPARATION AND SLIDE STAINING

Using the method of Rothvels and Siminovitch, (1978) and Worton and Duff, (1979) as described in Freshney (1983), the chromosomal content of cells in metaphase is determined. Briefly, 20 ug/ml of colchicine in PBS is added to cultures in their exponential growth phase, followed by incubated for 4-6 hours. Mitotic cells are detached by trypsinization (0.25% trypsin for 1 minute), and then incubated in 0.075M KCL at 37 C for 20 minutes. The cells are centrifuged (300xg, for 10 minutes) and fixed with 3 changes of 3:1 methanol:acetic acid. Cells in fixative are dropped from a height (splash method) onto clean slides, allowed to dry, then placed in an oven at 50C overnight. The dried slides are then stained with 3% Giemsa for 7 minutes.

For each treatment, 40 to 50 metaphase spreads are examined at each generation. The chromosome numbers were determined 48 hours, and up to 18

generations post exposure to the test materials. The results from the 48 hour, 4, and 18 generations are reported here. Because the initial presence of these materials is extremely cytotoxic to cells (Vaughan, *et al* 1991), the metaphase spreads obtained after the 48 hour exposure are significantly reduced (20 - 30 spreads per treatment). The percent mitotic cells and isolated nuclei containing fibers, therefore, were determined at this time point.

STATISTICAL ANALYSIS

All experiments are performed in triplicates with approximately four samples per generation for each treatment. The experimental data are evaluated by a one-way analysis of variance (ANOVA) or students two-tailed t-test, with the level of significance set at $p < 0.05$. A simple linear regression analysis was performed to determine the correlation coefficient (r).

3. RESULTS

Crocidolite asbestos and silicon carbide fibers have proven to cause cancer, namely, mesotheliomas in vivo. Both fibers are cytotoxic, reduce generation time, and induce cellular transformation in vitro within eight generations of exposure (Vaughan, *et al* 1991). This study explores the mechanisms of cell damage and the biological parameters that may be involved in the process of neoplastic transformation. It includes the quantitation of DNA and chromosome content up to 18 generations postexposure to crocidolite asbestos and SiCW. Earlier studies suggest an increase in cellular DNA and chromosome content might serve as an early marker for neoplasmic transformation in vivo. (Vanderlaan, *et al* 1983; LeMaire, *et al* 1989).

First, to determine if an increase in cellular DNA accompanies cellular transformation in vitro, mouse fibroblast cells in culture were exposed to the test materials as described in the materials and methods. As mentioned earlier, this cell line is non-tumorgenic, contact inhibited, and very sensitive to physical and chemical agents. It is, therefore, commonly used in assays to determine cellular transformation in vitro. Table 2 displays DNA concentration 10-12 generations postexposure to crocidolite and two different sizes of silicon carbide whiskers. The data from 3 experiments are averaged to give total DNA per cell and the percent increase as compared to control.

All samples tested are potent in inducing a significant increase in cellular DNA (Table 2). Asbestos, the smallest in diameter (0.36 M in diameters), was the most effective in inducing an increase in cellular DNA. The average DNA

TABLE 2. TOTAL DNA CONTENT, MOUSE CELLS (BALB/3T3)

Treatment	DNA/cell ($X10^{-11}$ g)	% Inc	n
Saline	2.49 (0.58)	____	45
Asbestos	4.37 (1.21)*	75%	18
SICW-2	3.54 (0.98)*	42%	29
SICW-1	3.48 (0.81)*	39%	32

( ) standard deviation

* significantly different from control ($P < 0.05$, ANOVA)

n=total number of samples after 3 experiments

content per cell for the saline-treated cultures was 2.49 x 10^{-11}g, while asbestos-treated cultures contained an average of 4.37 x 10^{-11}g. Asbestos, therefore, caused a 75 percent increase in total DNA per cell as compared to control. The man-made fibers, SiCW-1 (0.8 μM in diamters) and SiCW-2 (1.5 μM in diameters) caused approximately a 40% increase in DNA per cell. The results suggest the DNA of cells could be a possible target for solid-state carcinogens and the size of the fiber/whisker may determine its effectiveness. Also, an equal mass of asbestos will have more particles than SiCW-1 or SiCW-2. On a mass basis, asbestos, which is much smaller, caused a greater increase in DNA. As discussed previously (Vaughan, *et al* 1991), cellular response may depend more on the number of particles a cell encounters than on relative size. But the similarities between the DNA concentration of SiCW-1 and SiCW-2 suggest other factors, like chemical nature, may also be important.

Previous reports conclude cells in culture engulf these fibrous materials and after 24 hours are found in the cytoplasmic region to the cell (Vaughan, *et al* 1991, Wang, *et al* 1986). The cellular injury inflicted by these fibers are thought to be responsible for their cytotoxicity, and later results in the transformation of the surviving cells. In this study, the examination by light microscopy of the metaphase preparations shows these fibers not only in the cytoplasmic region of the cell, but in the nuclear region (Figure 1, 2, 3). Table 3 reports the percent mitotic cells and isolated nuclei containing fibers after 48 hours. Approximatley, 60 to 90 percent of metaphase spreads from the treated cultures contained fibers. Asbestos, the smallest in diameter, had the highest percentage of cells containing fibers (90%). Examination of the isolated nuclei from the same preparation, concluded that over 40% of the isolated nuclei

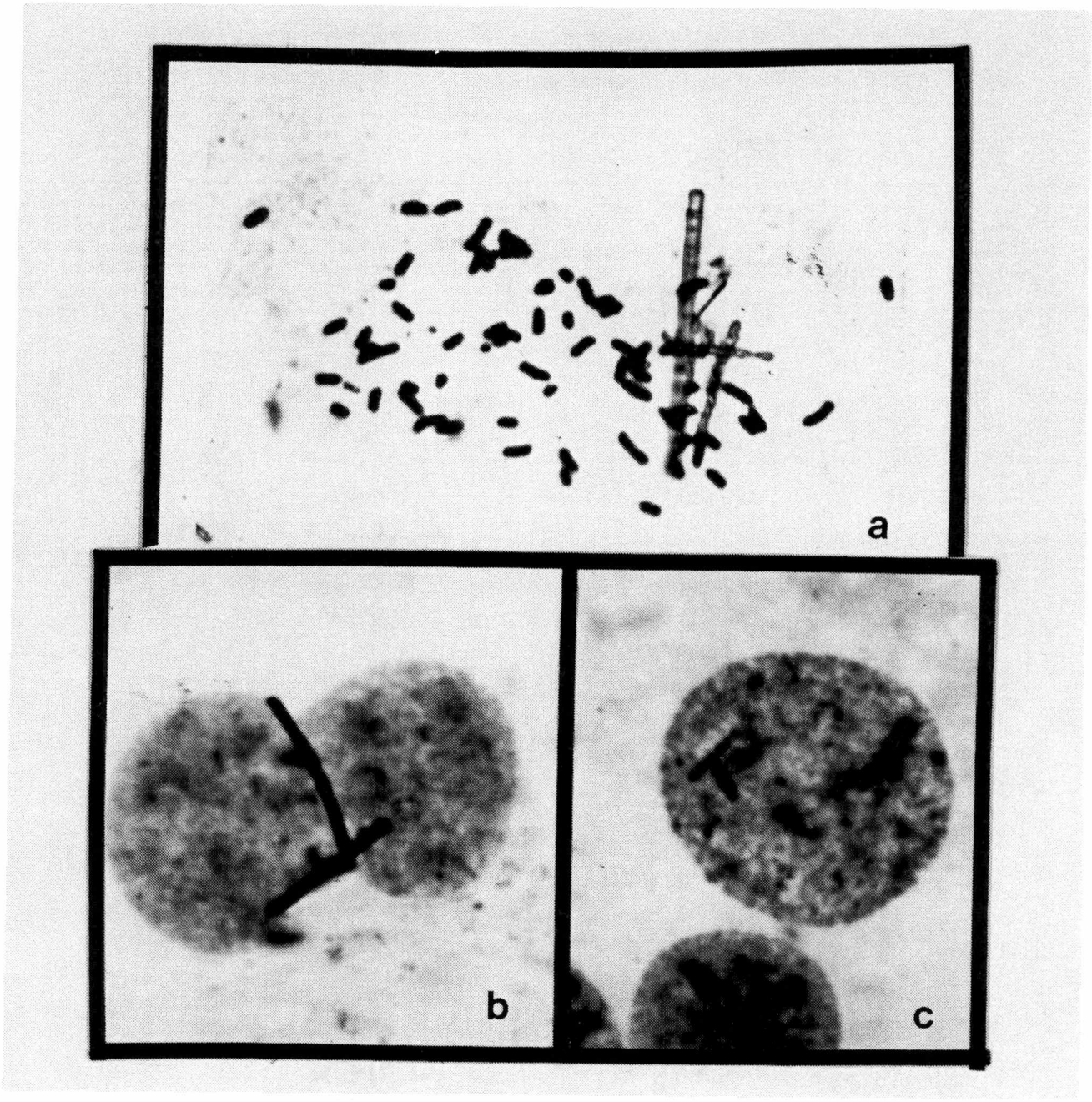


Figure 1. **Mouse fibroblast cells treated for 48 hours with silicon carbide (SiCW-2, 1.5 μM in diameter). a) Chromosomes attached to, or intertwine with fibers x12,000 b) Fibers appear to be preventing the separation of the nucleus, or splitting it into separate parts x12,000 c) Fibers attached to nuclear matrix x12,000.**

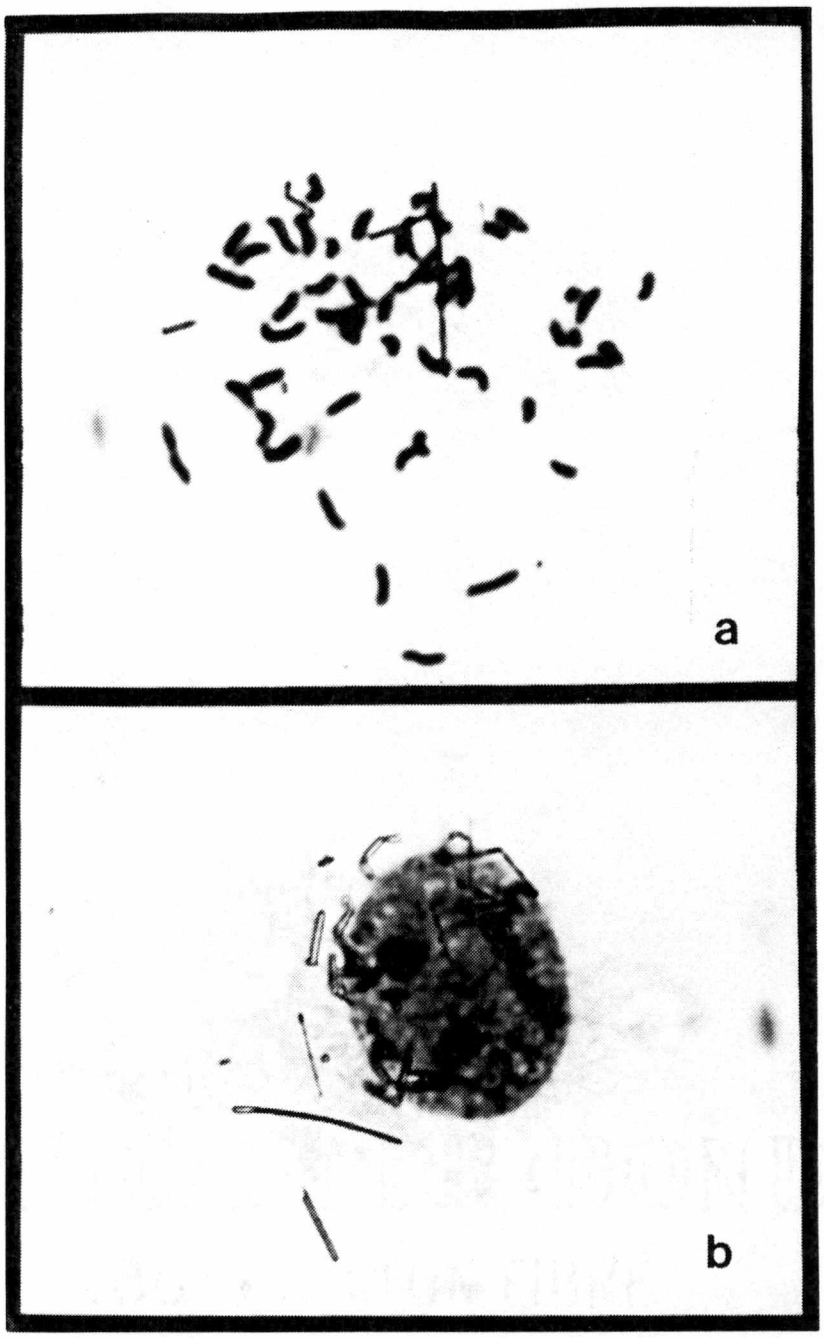


Figure 2. **Mouse fibroblast cells treated for 48 hours with silicon carbide (SiCW-1, 0.8 μM in diameter). a) Chromosomes appear to be entangled with, or attached to fibers x11,200 b) Fibers interacting with various parts of the nucleus x11,200.**

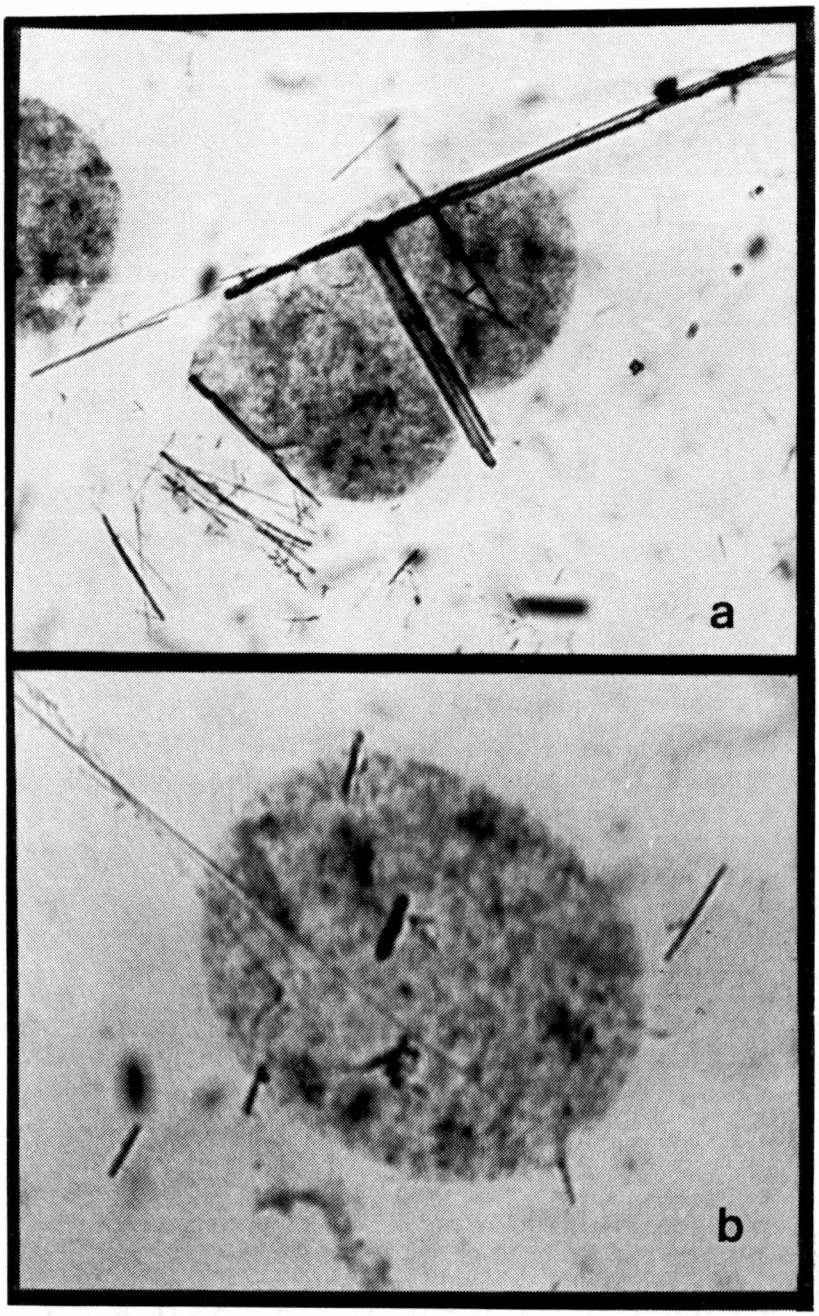


Figure 3. **Long, thin crocidolite asbestos fibers present in mouse cell nuclei. a,b) Fibers appear to be attached to the nuclear membrane x12,000 c) Chromosomes lying adjacent to, or attached to fibers x12,000 d) Long-thin fibers penetrating the nucleus x9,600.**

Figure 3. Continued

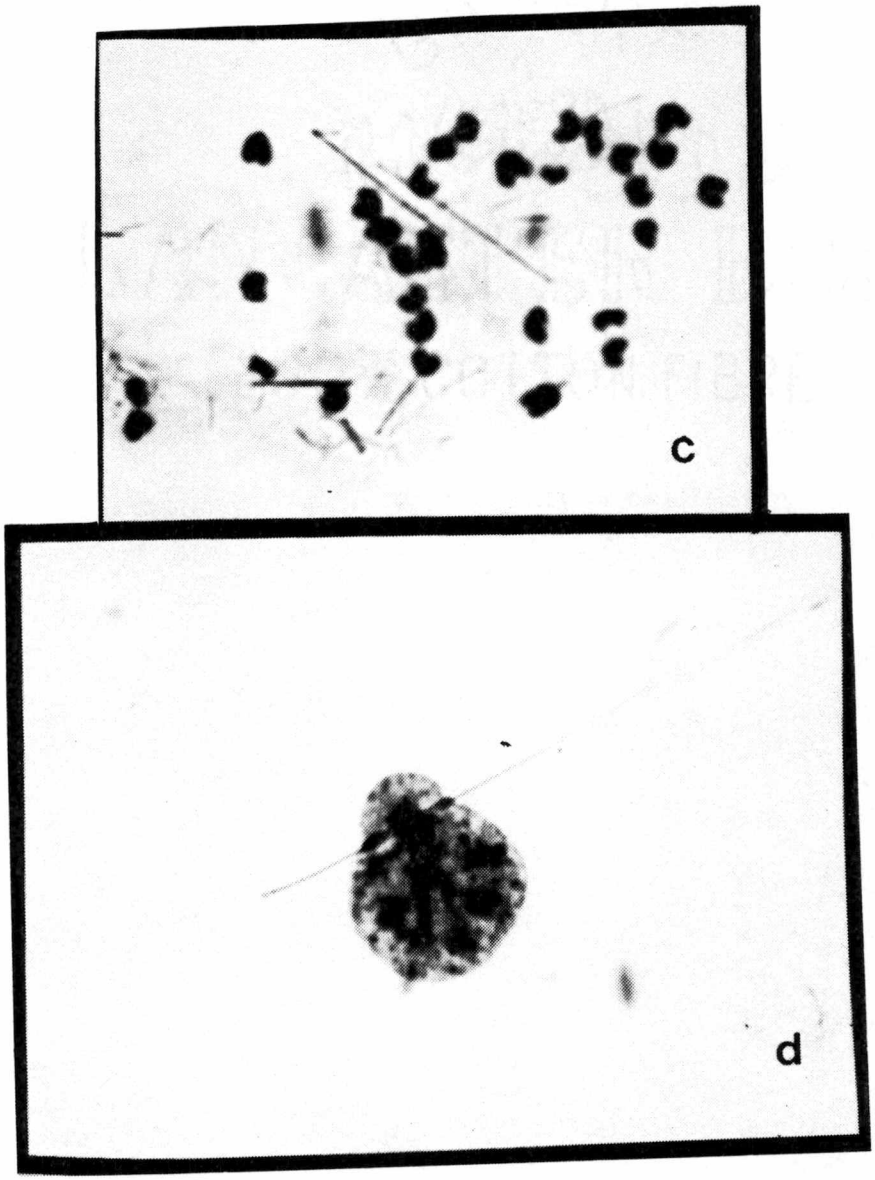


TABLE 3. PERCENT MOUSE CELLS CONTAINING FIBERS 48 HOURS POSTEXPOSURE TO CROCIDOLITE ASBESTOS OR SILICON CARBIDE

Treatment	% Mitotic Cells Containing Fibers	%Isolated Nuclei Containing Fibers
ASBESTOS	90% (n=52)	70%
SICW-1	_____(b)	60%
SICW-1	60% (n=38)	40%

n= number of metaphase spreads scored

(a)= a total of 100 nucle scored per treatment

(b)= a significant amount of metaphase spreads was not obtainable due to the cytotoxicity of SiCW-1 to determine the percent mitotic cells containing fiber, but isolated nuclei from the same preparation were determined.

scored contained this fibers. Again, asbestos had the highest percentage of isolated nuclei containing fibers (70%). SICW-2, the largest in diameter, had the least amount of nuclei containing fibers (40%), thus suggesting the importance of fiber size in determining the effectiveness of the fiber. All fiber/whiskers tested appeared to be physically or mechanically attached to various parts of the nucleus (Figures 1, 2, 3). They also caused an alteration in the morphology of the nucleus, and appeared to be either preventing their separation into daughter cells or literally splitting the nucleus into two separate parts (Figure 1). These specific interactions may play a role in the cytotoxic effects after 48 hours.

Chromosomal abnormalites are common change in carcinogenesis in vivo. Sincock, *et al* (1975) reported the induction of chromosomal changes in cells exposes to asbestos fibers in vitro. No change was observed with non-carcinogenic fibers. In this study, all samples tested were seen intertwined, attached to, or lying adjacent to metaphase chromosomes (Figures 1, 2, 3). Direct interaction may play a role in the development of chromosomal breaks, fragment, and deletions such as those seen by asbestos and glass fibers (Sincock, 1980; Huang, 1987). To determine whether the increased DNA content is indicative of ploidy, the chromosome number from mitotic spreads from mouse fibroblast cells were determined 48 hours, and up to 18 generations post exposure to fibers. The results from the 48 hour, 4, and 18 generations are reported here.

Table 4 shows the chromosomal abnormalites found in mouse fibroblast cells following exposure to crocidolite asbestos or silicon carbide. The data are presented as the range in the total chromosome number for each cell examined

TABLE 4. CHROMOSOMAL ABNORMALITES FOUND IN MOUSE FIBROBLAST CELLS FOLLOWING EXPOSURE TO CROCIDOLITE ASBESTOS OR SILICON CARBIDE

			CHROMOSOME NUMBER					OVERALL	
Exposure	Treatment	n	<30	30-50	50-70	70-80	>80	%Loss	%Gain
48 Hours	Control	64	0	21	39	4	0	0%	6%
	Asbestos	53	10	23	17	2	1	19%*	6%
	SiCW-1	UNDETERMINED							
	SiCW-2	43	3	19	13	4	4	7%	19%
4.0 Gen	Control	135	0	2	129	1	3	0%	2%
	Asbestos	92	3	7	71	1	10	3%	12%*
	SiCW-1	129	4	21	99	2	2	3%	3%
	SiCW-2	80	2	11	60	2	5	3%	9%
18.0 Gen	Control	109	0	31	77	1	0	0%	1%
	Asbestos	132	1	9	82	20	21	0%	31%*
	SiCW-1	120	0	19	80	13	18	0%	24%*
	SiCW-2	52	1	12	27	2	10	2%	23%

*significant difference as compared to control, $p<0.05$, t-test.

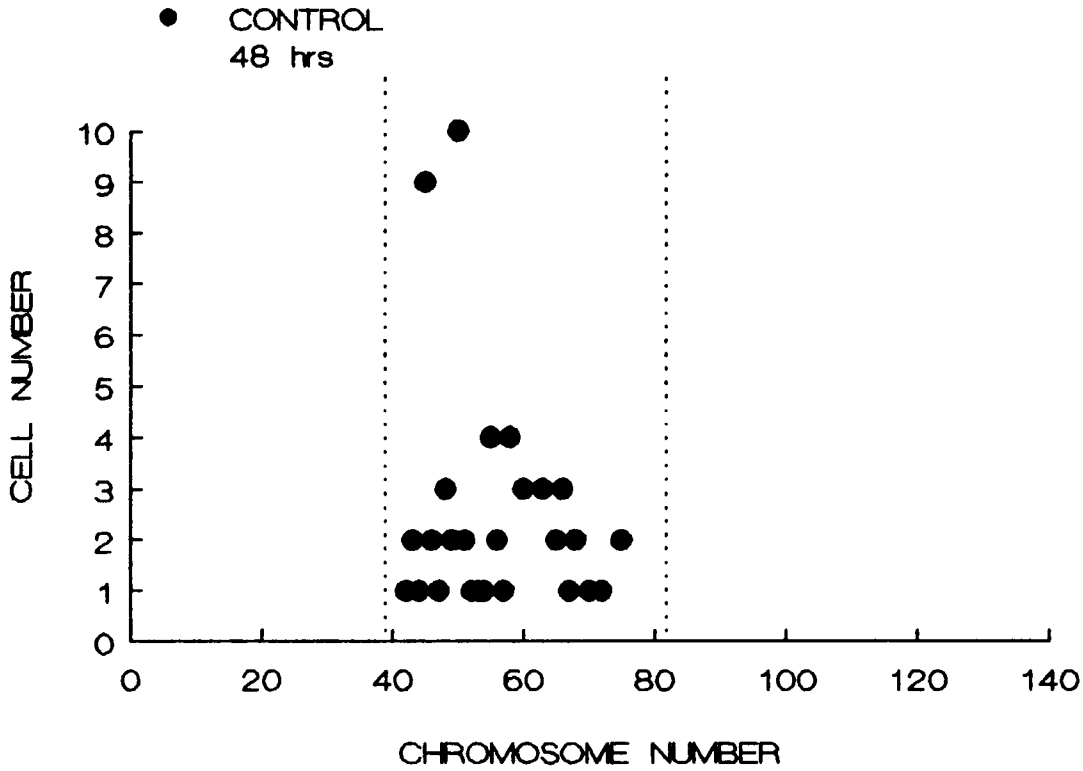
n= number of metaphase spreads

a= overall percent loss or gain in chromosomal number per treatment. (see text)

and the percent loss (<30) or gain (<70) in overall chromosomal content per treatment, as compared to the chromosome numbers of the control cultures. The percent loss or gain in chromosome number is determined by dividing the number of cells with chromosome numbers less than 30 or greater than 70 by the total number of cells examined. After 48 hours, there was a significant increase ($p<0.05$) in cells with chromosome number less than 30 in cultures treated with crocidolite asbestos (19%). Ten cells out of 53 had chromosome numbers less than 30. This significant decrease in chromosomal content is probably due the the initial cytotoxicity of this fiber. This decrease also supports the theory that the chromosomes attach to the fibers and disrupt cell division. SiCW-2 also showed an increased variation in chromosome number per cell as compared to the range of the controls, but was not significant when compared to the control values. Approximately 7% of the cells examined had chromosome number less than 30 and 19% of the cells had chromosome number greater than 70. Graphs showing the variation in chromosome number per cell after 48 hours are presentd in figure 4.0. Each graphs plots the total chromosomal content for each cell scored. The chromosome numbers for the control cultures ranged from 40 to 60. In cultures treated for 48 hours with asbestos, the range increased from 10 to 70 chromosomes per cell. Exposure to SiCW-2 resulted in a range of 15 to 135 chromosomes per cell. This alteration in chromosome number per cell may later be responisble for the cellular transformation of surviving cells.

As stated earlier, the primary goal of this research was to compare the long-term effects of crocidolite asbestos to that of silicon carbide using mammalian cells in culture. After 4 generations, cells treated with crocidolite asbestos showed a significant increase in cells with chromosomal contents above

Figure 4. **Graphs showing variation in chromosome number per cell 48 hours postexposure to fibers. Control, n=64, range 40 to 60; Asbestos, n=53, range 10 to 80; SiCW-2, n=43, range 15 to 153.**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48 hrs
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2
3
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5
6
7
8
9
10
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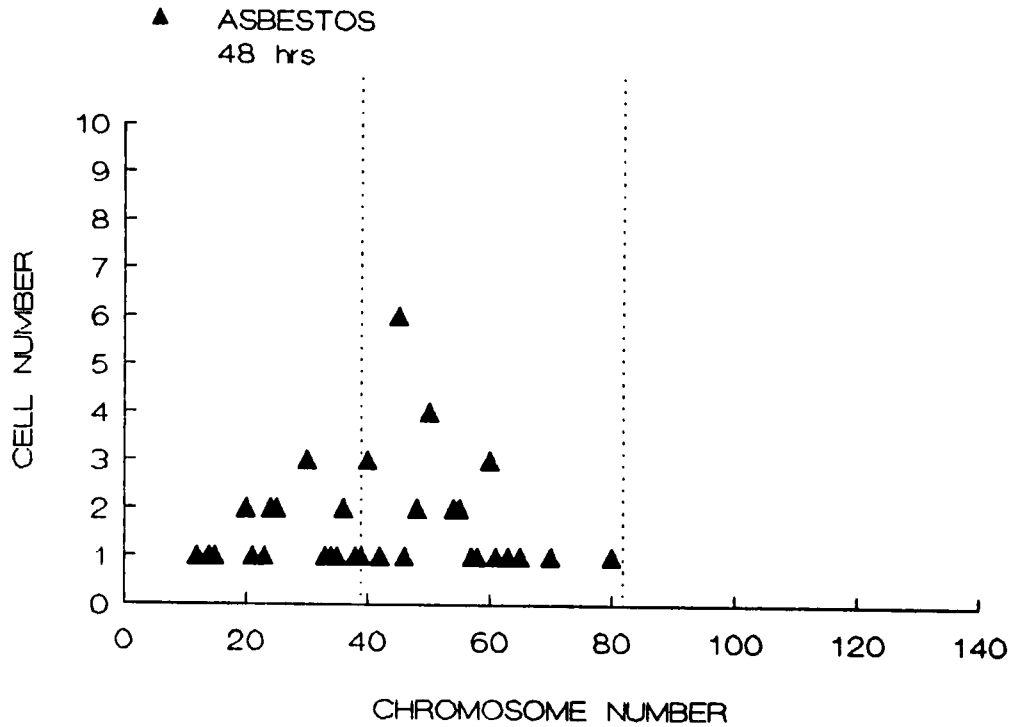
40
60
80
100
120
140
CHROMOSOME NUMBER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48 hrs
CELL NUMBER
CHROMOSOME NUMBER
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2
3
4
5
6
7
8
9
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20
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80
100
120
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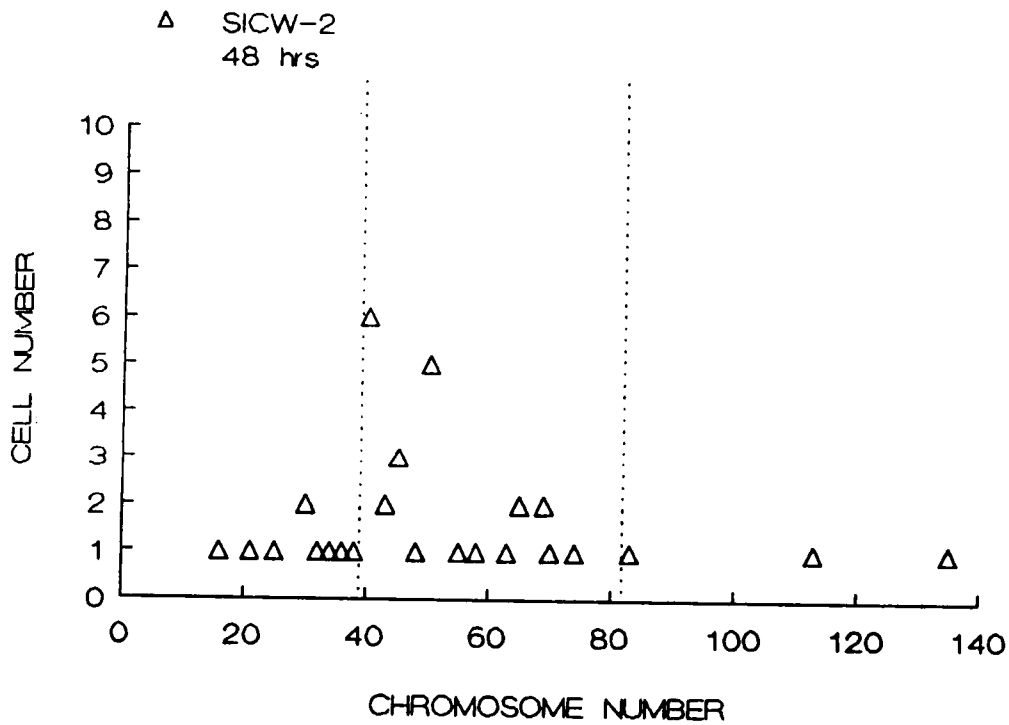
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1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100
120
140

70, see Table 4. Eleven cells out of 92 had chromosome numbers greater than 70, while 3 out of 92 had chromosome numbers less than 30. The man-made whiskers (SiCW-1 and SiCW-2) also showed an increased variation in chromosome number (Figure 5) , but was not significant at $p<0.05$. Graphs plotting the chromosomal content for each cell examined after 4 generations are presented in figure 6.0. There is an increase in the range of chromosome numbers from cultures treated with fibers as compared to the control cultures.

Surprisingly, after 4 generations crocidolite asbestos fibers were still present in about 10% of isolated nuclei examined (Figure 5d). The presence of these fibers, after the cells had been subcultured , provides an opportunity for the fibers to target the same cell or additional cells after the 48 hour exposure. Cultures treated with man-made fibers did not contain fibers at this time point.

Chromosomal abnormalities were still present 18 generations postexposure to test materials. Unlike the 48 hour exposure, these abnormalities resulted in an overall significant increase in total chromosomes per cell (Figure 7). Thirty-one percent of the cells treated with crocidolite asbestos had chromosome numbers above 70, see Table 4. Out of the 132 cells examined 41 had chromosome numbers greater than 70. Eighteen out of 120 (24%) of the cells treated with SiCW-1 had chromosome numbers above 70. SiCW-2 also showed an increase in chromosome number per cell (23%), but due to the small sample size was not significant at $p<0.05$ (t-test). Graphs showing the variation in chromosome number after 18 generations postexposure to fibers are presented in Figure 8. The variation which results in an overall increase in chromosome number per cell may be responsible for the increase in DNA seen

after 10-12 generation postexposure to fibers. There is a strong correlation between the increased DNA content and chromosome number (r= .0.96).

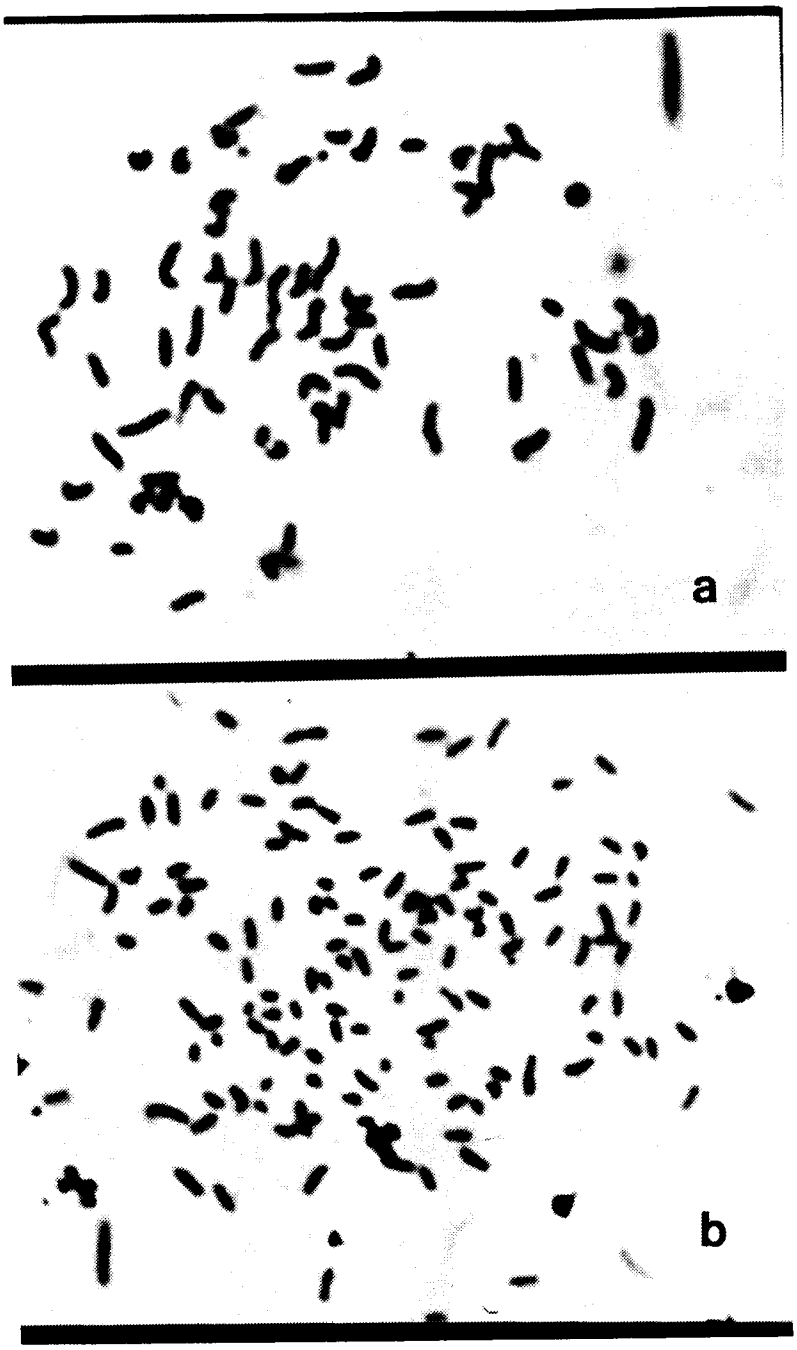


Figure 5. **Variation in chromosome number (n) 4 generations postexposure to test materials a) Control, n=65 x11,200 b) SiCW-1, n=105 x 8,000 c) SiCW-2, n=95, x 11,200 d) Presence of crocidolite asbestos 4 generations after the 48 hour exposure x11,200.**

Figure 5. Continued

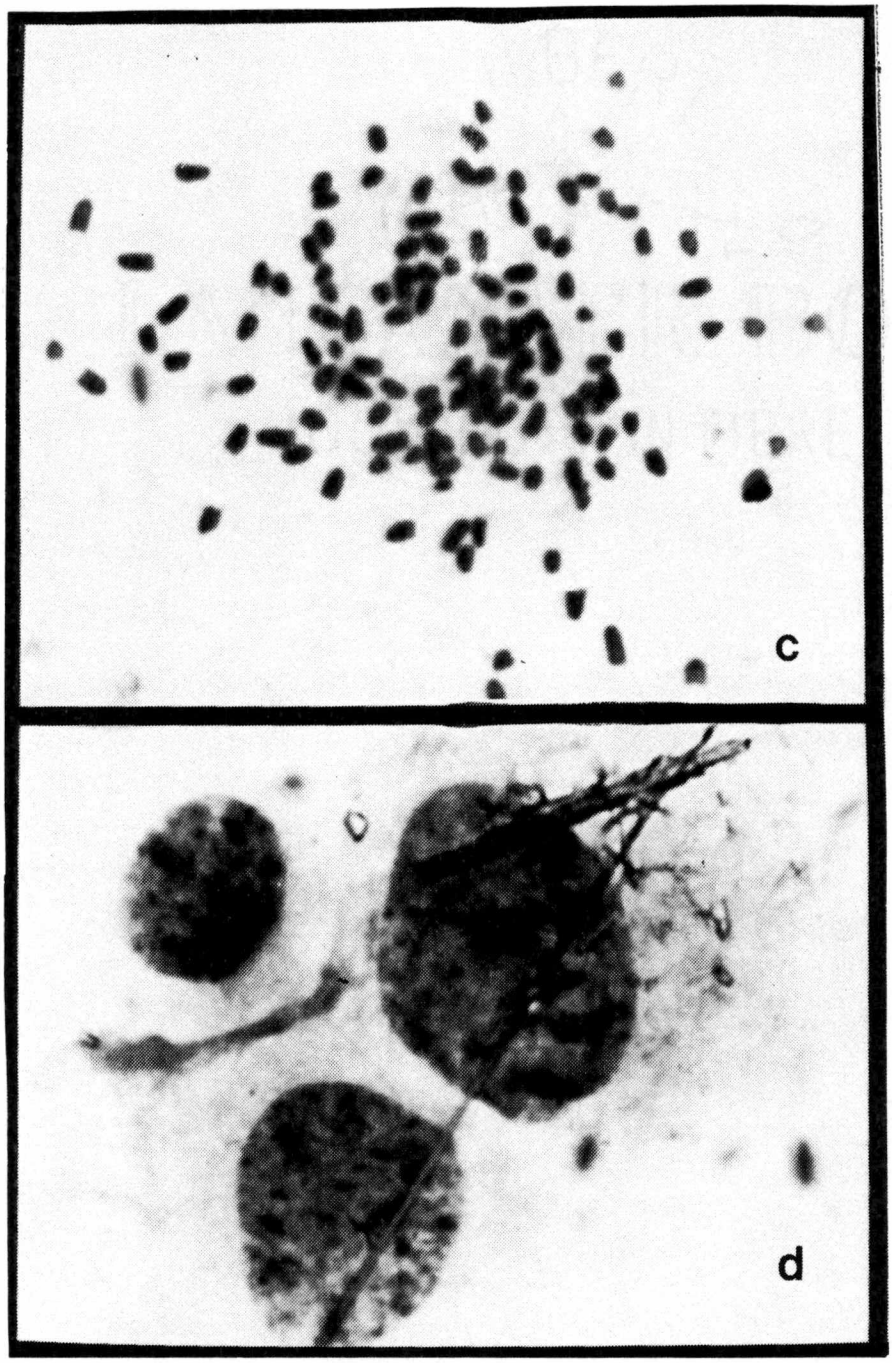
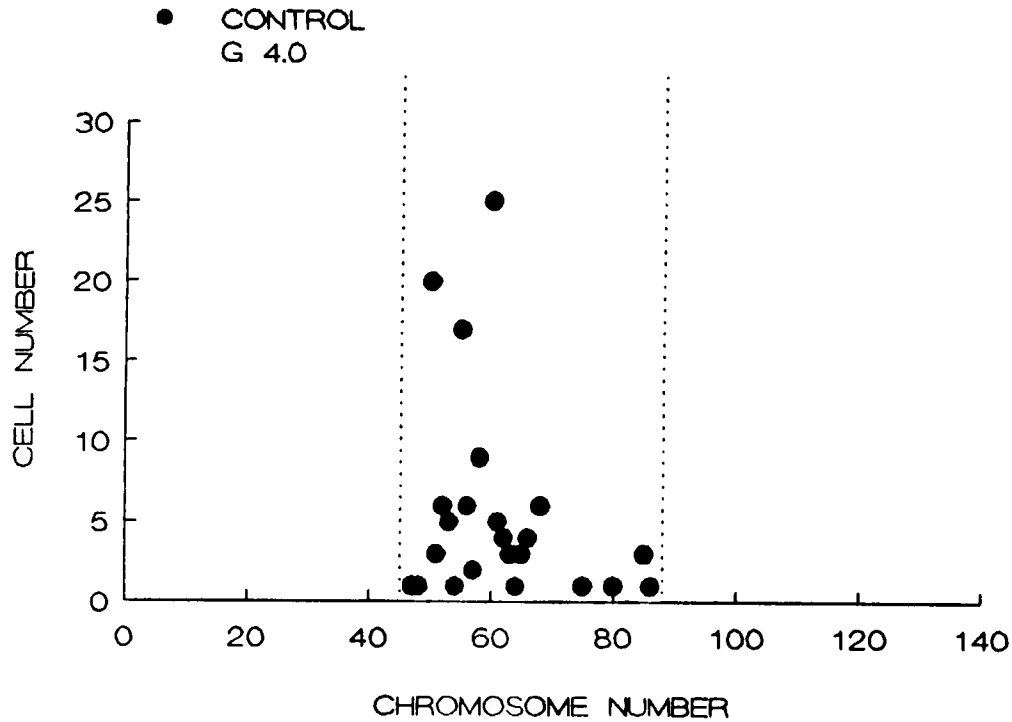


Figure 6. **Graphs showing variation in chromosome number per cell 4 generations postexposure to fibers. Control, n=153, range 45 to 85; Asbestos, n=92, range 12 to 110; SiCW-1, n=129, range 20 to 125; SiCW-2, n=80, range 20 to 100.**


CONTROL
G 4.0
CELL NUMBER
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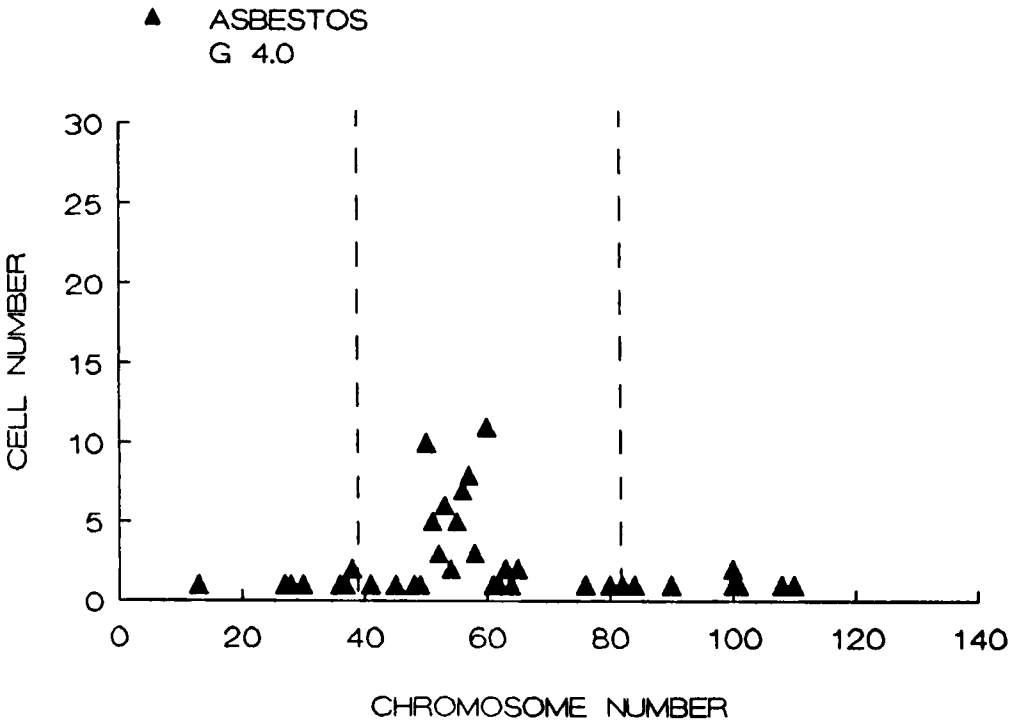
10
15
20
25
30
0
20
40
60
80
100
120
140


ASBESTOS
G 4.0
CELL NUMBER
CHROMOSOME NUMBER
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10
15
20
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30
0
20
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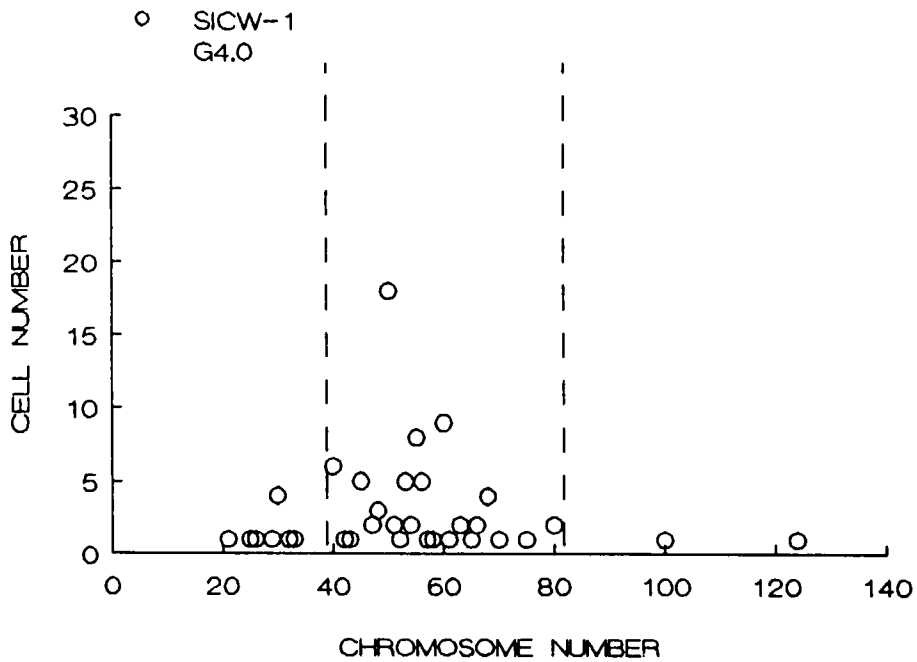
80
100
120
140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G4.0
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0
20
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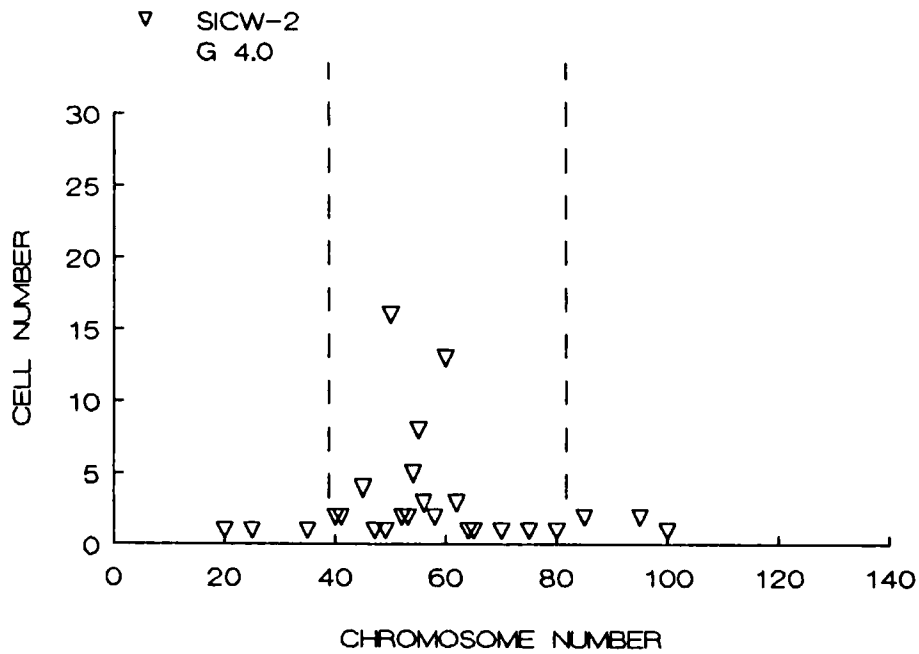
80
100
120
140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G 4.0
CELL NUMBER
CHROMOSOME NUMBER
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10
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80
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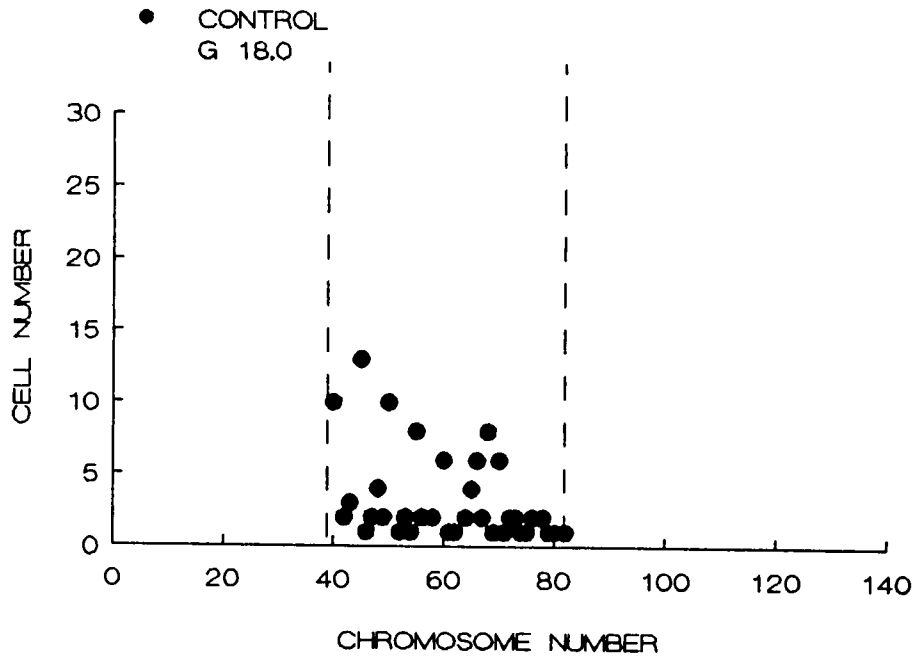
Figure 7. **Abnormal chromosomal content found in mouse cells 18 generations postexposure to crocidolite asbestos.**
a) Control, n=54 x11,200 b) Asbestos, n=125, x11,200.

Figure 8. **Graphs showing variation in chromosome number per cell 18 generations postexposure to fibers. Control, n=109, range 40 to 85; Asbestos, n=132, range 25 to 135; SiCW-1, n=120, range 35 to 130; SiCW-2, n=52, range 20 to 100.**


CONTROL
G 18.0
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25
20
15
10
5
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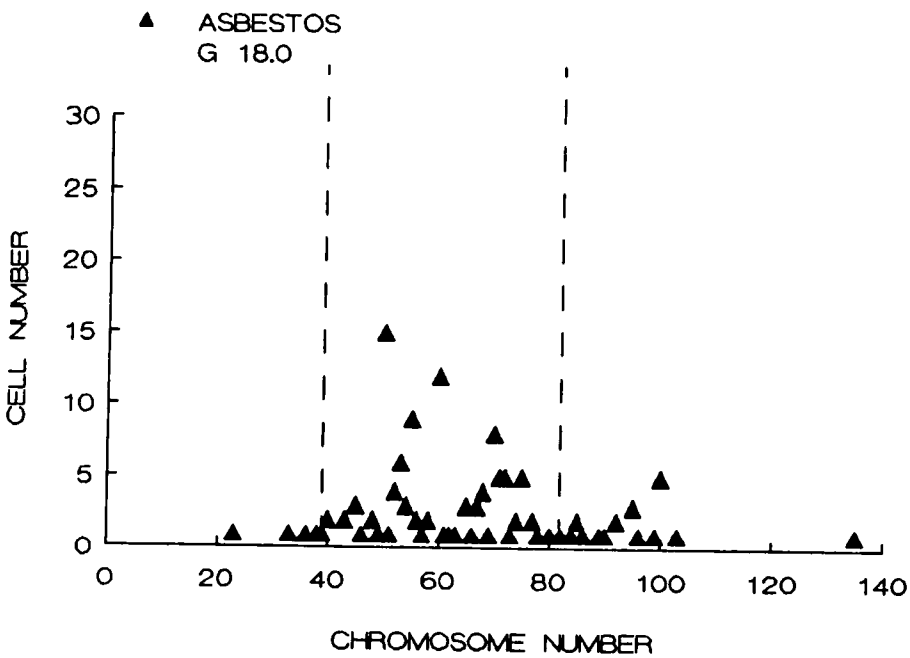
20
40
60
80
100
120
140
CHROMOSOME NUMBER


ASBESTOS
G 18.0
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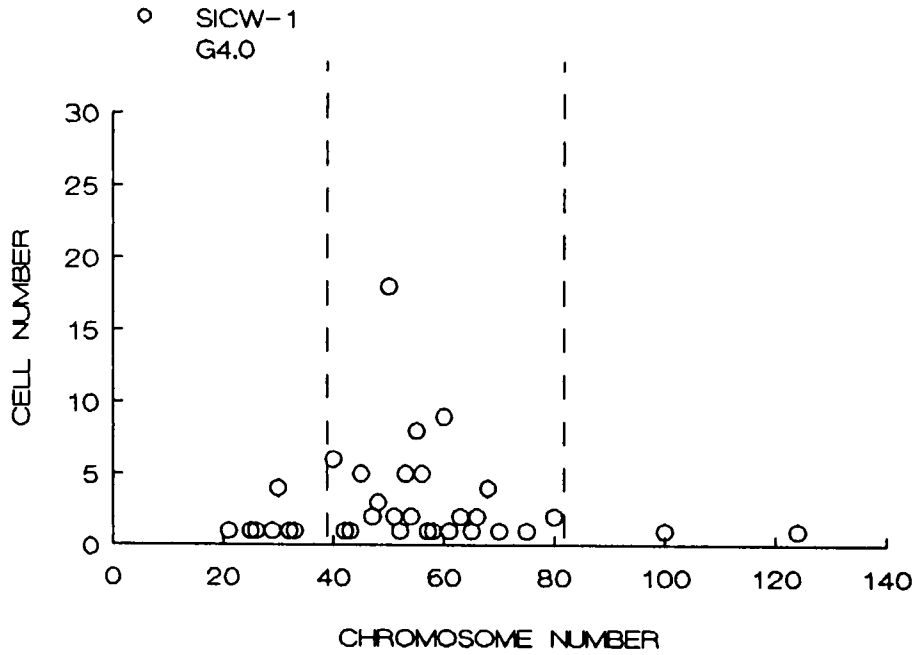
CHROMOSOME NUMBER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G4.0
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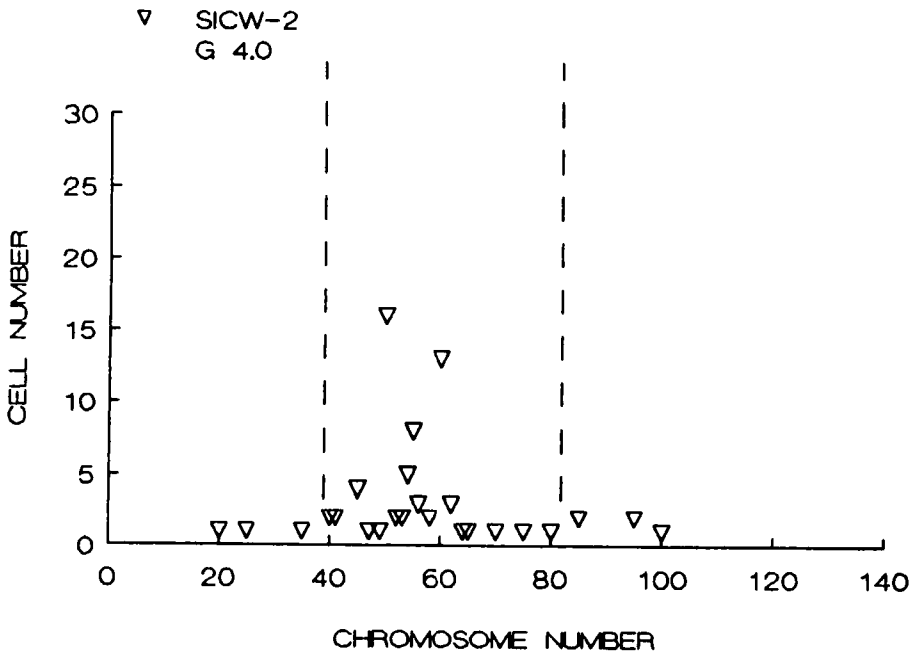
SICW-2
G 4.0
CELL NUMBER
CHROMOSOME NUMBER
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4. DISCUSSION

Natural and man-made fibers are carcinogenic. The biological effects of both have been studied extensively using cells in culture. But the mechanism associated with their carcinogenic ability is still unclear. One of the primary reasons is due to the lack of adequate in vitro tests to study the toxicity of solid-state materials.

Many factors may be involved in the carcinogenicity of fibrous materials. Experimental studies have concluded the fiber size and shape are responsible for the development of carcinomas and mesotheliomas in vivo and cellular transformation in vitro. (Stanton, *et al* 1977; Oshimura, *et al* 1984). The direct interaction between a fiber and a cell may be responsible for the neoplasmic conversion of a cell from the normal state.

Numerous carcinogens (i.e. chemicals) are thought to cause the initial conversion of a cell through the direct alteration of DNA. Different types of carcinogens are electrophiles that react convalently with DNA. These types of carcinogens are referred to as mutagens. There is no convincing evidence from in vitro studies that asbestos initiates tumors through direct interaction with DNA. Asbestos fibers have not shown to be mutagenic in bacterial or embryonic cells in culture (Oshimura, *et al* 1984). With the exception of Huang (1972), who reports that amosite, chrysotile, and crocidolite asbestos are mutagenic in chinese hamster lung cells. One of the primary problems involved in testing these fibrous materials for mutagenic activity is the ability of the cells in test system to engulf these fibrous materials. New in vitro test systems must be developed to determine the genotoxicity of solid-state particulates.

DNA, RNA, and chromatin have been shown to be absorbed to asbestiform minerals. Chang, *et al* (1990) concluded that the cytotoxicity of a fiber, correlates positively with the degree of macromolecular absorption of the fiber. Chrysotile has the greatest absorption, crocidolite, amosite and glass were significantly lower. The interaction between these fibers and cellular macromolecules could alter the biological functions of target cells, and result in genetic effects.

The results from this study suggest crocidolite asbestos and silicon carbide may interact with cellular DNA . This interaction (direct or indirect) may play a role in the cellular transformation of cells in vitro, which is suggestive of carcinogenicity. Within 10-12 generations of exposure, all types of fibers induced a significant increase in cellular DNA. (see Table 2). Asbestos induced a 75 percent increase in cellular DNA, while SICW-1 and SICW-2 induced approximately a 40 percent increase. Both types of fibers caused an increase in DNA and the size of the fiber, number, or chemical nature appears to determine the effectiveness. Nevertheless, the increase DNA content correlates with studies reporting an increased amount of cellular DNA content found in many tumors.

Human cancers can result from several different mechanisms. One specific mechanism involves the numerical and/or structural variations of normal chromosomal content. Experimental studies have shown both natural and man-made fibers induce chromosomal aberrations (i.e. breaks, gaps, inversions) in vitro (Wang, *et al* 1986; Huang, 1987; Oshimura, *et al* 1984; Price-Jones, *et al* 1980). Sincock, *et al* (1977) also reports that all types of abestos fibers give rise to high levels of polyploids in vitro, leaching or milling reduces the effect.

Direct physical and mechanical interaction between fibers and cell nuclei were observed (Figures 1,2,3) by light microscopy and thought to be responsible for variation in chromosome number per cell (aneuploidy) 18 generations postexposure to fibers. After 48 hours, all three sizes of fibers were seen entangled with, or attached to metaphase chromosomes (60 - 90 %) (see Table 4 and Figures 1a, 2a,). This interaction could occur during the movement of the chromosome during prometaphase and metaphase. The events that occur during the separation of the chromosomes between daughter cells involves microtubule elongation from two centrosomes, chromosome attachment to microtubules, microfiliaments, and other structural components that may play a role in causing the interaction between the chromosomes and the fibers (Wang, *et al* 1988; Juarand, *et al* 1989). Wang, *et al* (1988) also studied the interaction between metaphase chromosomes and chrysotile or crocidolite asbestos on mesothelial cells in culture after a 48 hour exposure. Chrysotile which is positively charged caused greater chromosomal change than crocidolite which is negatively charged. Suggesting the difference in the surface properties may play a critical role in the interactions between the fibers and the chromosomes. The examination of mitotic spreads in this study does not support this theory (Table 3, Figure 1b, 2b). Crocidolite which is negatively charged caused a greater chromosomal change than both SiCW-1 or SiCW-2, which are positively charged (see Table 2, 4). After the 48 hour exposure, 90% of the mitotic spreads examined from cultures treated with crocidolite asbestos contained fibers. Approximately, 60% of the cultures treated with the man-made fibers contained fibers. This suggest surface properties like charge are important, but other factors like size, shape, number and the chemical difference between

natural and man-made fibers must not be ignored. All of these interactions may play a role in the chromosomal aberrations and aneuploidy commonly seen after a 48 hours exposure to asbestiform materials. The earlier observation of increased DNA suggest the physical or mechanical interaction between the chromosomes and the fibers may be an important mechanism responsible for their carcinogenicity.

Some researchers suggest the variation in chromosome number per cell noted after the initial exposure is due to the cytotoxic effects of the fibers, but are may not be effective in causing any long-term cytogenetic effects in vitro (Price-Jones, *et al* 1980). This study concludes exposure to natural or man-made fibers later results (18 generations) in a significant increase in chromosomal content per cell. (Table 4, Figure 7). SiCW-2 and SiCW-1 showed approximately a 24 percent increase in chromosome number per cell, while crocidolite showed a 31 percent increase. As mentioned earlier, this increase may be due to the interaction between the fibers and the cells during the 48 hour exposure. (Figure 1a, 2a). The chromosomal content of mouse fibroblast cells treated with fiber/whiskers exceeds the control values after 18 generations (see Figure 8). These findings correlate with experimental studies suggesting abnormal chromosomal content (ploidy) in many tumors (mesotheliomas).

In summary, solid-state materials, like asbestos, are currently classified as epigenetic carcinogens. The mechanism of action is unknown, but experimental evidence suggest these materials act indirectly with the DNA or chromosomes of target cells. Crocidolite asbestos and silicon carbide fibers have been proven to cause mesotheliomas in humans and experimental studies

using animals. Because the latency period in the development mesotheliomas is about 20 years, in vitro studies are used to determine the long-term carcinogenic ability of natural or man-made materials. Although animal studies are important, they are also expensive, time consuming, and not able to provide sufficient information on the carcinogenicity of fibrous materials. In vitro cellular transformation assays, which are inexpensive and less time-consuming, are commonly used to predict the long-term biological effects of a potential carcinogen. Previous studies have shown both natural and man-made fibers transform cells in vitro. (Vaughan, *et al* 1991) This study suggest cellular transformation in vitro , is also accompanied by an increase in cellular DNA and chromosome content, thus suggesting the genotoxic ability of natural and man-made fibers in vitro. A battery of in vitro cytogenetic test using mammalian cells in culture may be useful in suggesting the long-term biological effects of a potential solid-state carcinogen. This study also supports previous studies suggesting the mechanism of carcinogenicity of natural and man-made fibers may involve the direct interaction between fibers and target cells (Wang, *et al* 1988, Jaurand, *et al* 1990). It also provides additional information that the initial interaction between the fibers and cells later results in a significant increase in the DNA (mutations, deletions) and chromosomes (breaks, fragments, altering cell cycle) in vitro. The cellular responses perceived in this study are common for many genotoxic carcinogens. These findings, therefore, are in support of the theory suggesting natural and man-made fibers/whiskers are in fact genotoxic carcinogens.

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